Brazilian Journal of Microbiology (2010) 41: 321-328
ISSN 1517-8382

# REGENERATED EXTRACELLULAR $NH_4^+$ AFFECTS THE MOTILE CHEMOSENSORY RESPONSES OF BATCH-CULTURED *OXYRRHIS MARINA*

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Submitted: June 01, 2009; Returned to authors for corrections: June 30, 2009; Approved: October 06, 2009.

## ABSTRACT

Regenerated extracellular $NH_4^+$ in laboratory batch-cultures of the heterotrophic marine microzooplankter *Oxyrrhis marina* affects the strength and consistency of chemotaxes elicited by synthetic and biogenic chemoattractants. The ecological relevance of experiments with batch-cultured *O. marina* and limitations of the microcapillary assay for the study of chemosensory behaviours are discussed.

**Key words:** Chemosensory, Chemotaxis, Chemotaxes, *Oxyrrhis marina*

Chemotaxis, the directional movement of cells or organisms in response to chemical stimulation is an ancestral and essential physiological response (18) first described by W. Pfeffer in 1884 following observations of the attraction of fern sperm to the ova. Since then the phenomenon has been observed in cell types ranging from amoeboid slime molds (4) and bacteria (1) to mammalian macrophages (10). In the field of microbial marine ecology, motile chemosensory responses to dissolved chemical stimuli have been documented in marine bacteria (13), autotrophic algae (26) and cryptophytes (17) and are understood to assist in nutrient acquisition and avoidance responses (e.g. to noxious compounds). It is suspected that some symbiotic marine microbes (e.g. the dinoflagellate *Symbiodinium microadriaticum*) might use chemotaxis to help locate specific hosts (22); another dinoflagellate species - *Crypthecodinium cohnii* - shows strong positive chemotaxis towards polysaccharides derived from its specialist prey species – the marine microalga *Porphyridium* (29). Given the specificity of *S. microadriaticum* and *C. cohnii*, it is reasonable to infer that the chemosensory apparatus and responses of both could be highly refined. For heterotrophic protozoa that consume a wider variety of prey items - hereafter focusing on the marine microzooplankter *Oxyrrhis marina* - it has been postulated (9) and demonstrated (19, 21) that chemotaxis can enhance the overall efficiency of prey location. Pertinently, it has long been assumed that *O. marina* must possess chemosensory apparatus with an affinity for dissolved chemical stimuli (11, 28). However, despite being employed as a model organism for well over a decade (for recent review [20]) the biochemical basis of any such apparatus remains poorly investigated. Instead, studies designed to explore chemotaxis have tended to record high variability in the sensitivity and consistency of chemotaxes observed in *O. marina*. For example, in 1985 it was reported that *O. marina* only showed motile chemoresponses towards a shrimp extract (that elicited positive chemotaxis in other heterotrophic protozoa) 72 hours after being transferred from batch-culture with the diatom *Phaeodactylum*, to culture with the microalgae

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*Dunaliella salina* (27). At this time, it was proposed that the chemosensory apparatus of *O. marina* might only be activated when cells are maintained using certain prey species (27); however no explanation as to why this may be is given. Similarly, a recent study found variation in threshold detection limits (the lowest concentration of a stimulant required to elicit chemotaxis) and inconsistencies between the compounds that evoked the strongest and weakest chemosensory responses in batch-cultured *O. marina* (19).

The aim of experimental work reported here was to simply explore the importance of batch-culture conditions – specifically the effect/s of regenerated extracellular $NH_4^+$ - on the motile chemosensory responses of *O. marina*. It should first be noted that *Oxyrrhis marina* is a heterotrophic (non-photosynthetic) species in which losses of cell-carbon during respiration are coupled with the regeneration of nitrogen – as extracellular $NH_4^+$ (5, 14). Secondly, when used in laboratory studies, *O. marina* is frequently maintained in batch-culture on autotrophic prey (e.g. the microalgae *Dunaliella primolecta*) that assimilate $NH_4^+$ during photosynthesis (12). Thirdly, previous work has shown that synthetic and biogenic sources of $NH_4^+$ elicit positive chemotaxis in *O. marina* (19). On the basis of the latter consideration, it was reasoned that accumulation of regenerated extracellular $NH_4^+$ in prey-deplete batch-cultures of *O. marina* (Fig. 1) could affect the strength of motile chemosensory responses elicited by alternative stimuli (particularly if they are assayed at lower concentrations than ambient $NH_4^+$). To investigate this possibility, a standard microcapillary assay (2) was used to measure the motile chemosensory responses elicited by known chemoattractants (19) in *O. marina* that were removed from a batch-culture (Batch-culture study, Table 1a) as it progressed from a prey-saturated, low $NH_4^+$ condition (Fig. 1b) to a prey-depleted, high $NH_4^+$condition (Fig. 1c). A second experiment was then undertaken to determine whether chemotaxis in *O. marina* could be suppressed and recovered; first by incubating cells in seawater-based $NH_4Cl$, and then by transferral of the same cells back into $NH_4^+$-free seawater ($NH_4Cl$ addition and removal experiment, Table 1b).

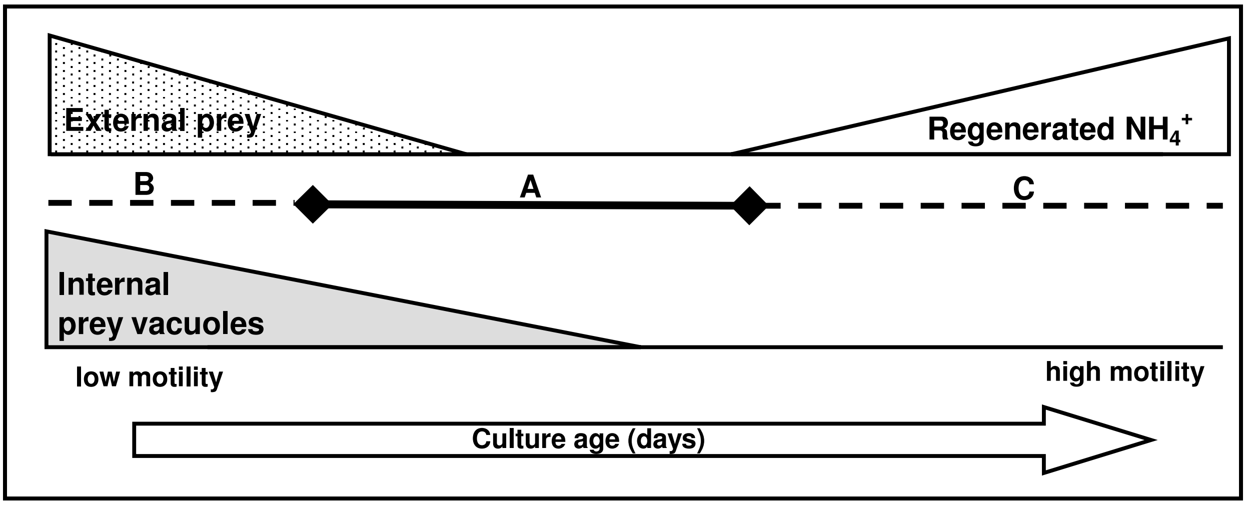


**Figure 1.** Schematic of the 'window of chemotaxis' (A) - when measurements of chemotaxes in batch-cultured *Oxyrrhis marina* are not affected by regenerated $NH_4^+$. Dashed lines indicate sampling intervals when (B) low cell motility (owing to the presence of multiple food vacuoles inside *O. marina*) or (C) concentrations of regenerated $NH_4^+$ (that accumulate in the absence of photosynthetic prey) affect measurement of motile chemosensory responses. Note that other regenerated / chemical compounds (e.g. prey-associated toxins) could accumulate and affect motile chemosensory responses in the same way.

**Table 1.** Response Indices elicited by chemoattractants in *Oxyrrhis marina* during (A) batch-culture and (B) $NH_4Cl$ addition and removal studies: □ = cell-free culture filtrate; ○ = 100µM arginine (unless otherwise specified); ◇ = 100µM $NH_4Cl$. Open symbols = response index of 1.0 (response not different from that elicited by $NH_4^+$-free seawater); Filled symbols = response index > 2.0 (positive, measurable chemotaxis).

**A**

| | Culture Age (day) 1 | 2 | 4 | 6 | 8 |
|---|---|---|---|---|---|
| External prey (cells $mL^{-1}$) $3.5 \times 10^5$ | $2.0 \times 10^4$ | $5.1 \times 10^3$ | $<1.0 \times 10^2$ | $<1.0 \times 10^1$ | |
| *O. marina* biovolume (pL $cell^{-1}$) | 4.96 | 2.60 | 1.77 | 1.55 | 1.50 |
| Regenerated $NH_4^+$ (µM) | <1.0 | 8.0 | 26.0 | 66.0 | 123.0 |
| Time after transfer to $NH_4^+$-free seawater (hrs) | Response Index | | | | |
| 3 | □ ○ ◇ | ■ ○ ◇ | □ ○ ◇ | □ ○ ◇ | □ ○ ◇ |
| 6 | □ ○ ◇ | ■ ○ ◇ | □ ○ ◇ | □ ○ ◇ | □ ○ ◇ |
| 12 | ■ ● ◆ | ■ ● ◆ | □ ○ ◇ | □ ○ ◇ | □ ○ ◇ |
| 24 | ■ ● ◆ | ■ ● ◆ | □ ○ ◇ | □ ○ ◇ | □ ○ ◇ |
| 48 | ■ ● ◆ | ■ ● ◆ | □ ○ ◇ | □ ○ ◇ | □ ○ ◇ |

**B**

| | External $NH_4Cl$ (µM) 0 | 5 | 10 | 20 | 50 |
|---|---|---|---|---|---|
| Arginine (µM) | Response Index (in situ) | | | | |
| 100 | ● | ○ | ○ | ○ | ○ |
| Arginine (µM) | Response Index (3hr after transfer to $NH_4^+$-free seawater) | | | | |
| 1 | ○ | ○ | ○ | ○ | ○ |
| 10 | ● | ○ | ○ | ○ | ○ |
| 100 | ● | ● | ○ | ○ | ○ |
| 500 | ● | ● | ○ | ○ | ○ |
| 1000 | ● | ● | ○ | ○ | ○ |

In all experimental work, monoxenic batch-cultures of *Oxyrrhis marina* Dujardin (CCAP 1133/5) were maintained using exponential phase *Dunaliella primolecta* Butcher (CCAP 11/34) grown at 18°C (±1°C) in an 18:6 hour light:dark cycle (180µmol photons $m^{-2}$ $s^{-1}$) using modified (440µM nitrate) *f*/2 media (15). Measurements of chemotaxis were made using a modification of Adler's (1973) micropipette technique (2, 19). Briefly, disposable 2µL microcapillary tubes (CAMAG Biosciences, UK) were filled via capillary action with a test or control ($NH_4^+$- free seawater) solution and placed in Petri dishes containing *O. marina* ($4 \times 10^4$ cells $mL^{-1}$) that had been washed and resuspended (for 3 hours) in $NH_4^+$- free seawater. After 2 minutes counts of the number of *O. marina* observed inside experimental (containing a test solution) or control tubes (5 tubes per assay) were made using light microscopy. These counts were used to derive a ($t_2$min) response index as: mean number *O. marina* experimental $tube^{-1}$ ÷ mean number *O. marina* control $tube^{-1}$. All response indices were rounded (up or down) to the nearest integer such that, in this report: a response index of 1.0 reflects no chemotaxis (the motile response elicited by a test chemoattractant was no different from that elicited by the seawater control solution); a response index of 2 (or higher) is indicative of positive, measureable chemotaxis (Table 1).

For the batch-culture study (Table 1a), a prey-saturated (high prey, low $NH_4^+$; Fig. 1) culture was established by mixing *O. marina* with *D. primolecta* to achieve starting (day 1) cell densities of $3.0 \times 10^4$ cells $mL^{-1}$ and $3.5 \times 10^5$ cells $mL^{-1}$ respectively. The culture flask was then wrapped in aluminium foil to prevent photosynthesis ($NH_4^+$- uptake) by uneaten *D. primolecta* and to allow accumulation of regenerated extracellular $NH_4^+$. Cell numbers and biovolumes for *O. marina* and *D. primolecta* were monitored for 8 days using an Elzone 282 PC particle analyser (Particle Data Inc); this enabled estimation of the size (biovolume) of individual *O. marina* as they progressed from a vacuole-replete (containing prey multiple vacuoles) to a vacuole-deplete (no prey vacuoles) status. At each sampling interval, the concentration of regenerated extracellular $NH_4^+$ in the batch-culture was estimated according to the method of Holmes et al., (16). The motile chemosensory responses elicited in *O. marina* (3, 6, 12, 24 and 48 hours after transfer of cells from the

experimental batch-culture to $NH_4^+$- free seawater) by i) cell-free filtrate from the original culture flask, ii) 100µM arginine and iii) 100µM seawater-based $NH_4Cl$ (Table 1a) were then assayed. It should be noted that the maximum (48 hour) transfer time was chosen largely because after this time, levels of $NH_4^+$ in the $NH_4^+$-free seawater began to increase (owing to continuing respiration and $NH_4^+$-regeneration from transferred *O. marina*). For the second $NH_4Cl$ addition and removal experiment (Table 1b), chemotactic *O. marina* (showing positive chemotaxis towards 100µM arginine) were incubated (for 3 hours) in 0µM, 5µM, 10µM, 20µM or 50µM (final concentration) seawater-based $NH_4Cl$. The motile chemosensory responses of *O. marina* to arginine were assayed with the microcapillary technique i) 'in situ' (using a 100µM arginine test solution) and ii) 3 hours after washing and transfer of cells back into $NH_4^+$-free seawater (using 1µM, 10µM, 100µM, 500µM or 1mM arginine test solutions, Table 1b).

In the batch-culture study, measurements of chemotaxis were affected by the satiation status of *O. marina* and – as hypothesised - concentrations of regenerated extracellular $NH_4^+$ (Table 1 and Fig. 1). *O. marina* removed from the batch culture on day 1 were visibly vacuole-replete (containing >5 *Dunaliella* prey vacuoles; mean *O. marina* biovolume 4.96 pL $cell^{-1}$) and displayed very low motility. Measurable motile chemosensory responses (response indices > 2.0) to i) cell-free culture filtrate, ii) 100µM arginine and iii) 100µM $NH_4Cl$ solutions were only observed 12, 24 and 48 hours after transfer of these (day 1) *O. marina* to $NH_4^+$- free seawater – after which time many divided, yielding smaller (2.0 pL $cell^{-1}$) motile daughter-cells. *O. marina* removed from the batch-culture on day 2 contained fewer (1-5) *D. primolecta* prey vacuoles, were small (≈ 2.6 pL $cell^{-1}$) and visibly more motile. However - despite their increased motility – they only responded to arginine and $NH_4Cl$ chemoattractants 12 hours after transfer to $NH_4^+$-free seawater (Table 1a). Interestingly, positive chemotaxis was elicited by cell-free culture filtrate (≡ 8µM $NH_4^+$) just 3 hours after transfer. As shown previously (19) these results demonstrate that regenerated $NH_4^+$ (and other non-quantified biogenic stimuli [e.g. amino acids leaked from conspecifics]) elicit strong, positive chemotaxis in *O. marina*. Furthermore, they illustrate that, following transfer between different culture conditions, a recovery or adjustment period is required before measurable chemotaxes are inducible in *O. marina*. Importantly, the length of this adjustment period appears to be shorter for biogenic chemoattractants – possibly because they comprise a mixture of stimulatory compounds that act synergistically on any chemosensory apparatus involved in the propagation of chemotaxis in *O. marina*. It is significant that – even after a 48 hour recovery period in $NH_4^+$-free seawater (Table 1a) - no measurable motile chemosensory responses were observed in *O. marina* removed from the batch-culture on days 4, 6 or 8 (extracellular $NH_4^+$ concentrations of 26µM, 66µM and 123µM respectively). It might be that chronic exposure of *O. marina* to regenerated $NH_4^+$ (and other biogenic compounds that accumulate in the batch-culture system) results in long-term acclimatisation to ambient chemical conditions (8) such that cells no longer respond to $NH_4^+$. The sensitivity of motile chemosensory responses may be downregulated (e.g. 24) or even the repressed at the genetic level in order to maintain intracellular homeostasis. Alternatively it might be that chronic exposure of *O. marina* to $NH_4^+$ and/or additional biogenic chemical stressors has adverse effects on aspects of cell physiology (e.g. causing damage to cellular organelles) that regulate cell motility and/or the propagation of chemosensory responses.

In the second $NH_4Cl$ addition and removal experiment (Table 1b), the motile responses of chemotactic *O. marina* (showing positive chemotaxis towards 100µM arginine) were clearly affected by ambient (5µM-1000µM) concentrations of $NH_4Cl$. Despite being between 2 and 20-fold more concentrated than 'in situ' concentrations of $NH_4Cl$, the 100µM arginine test solution still failed to elicit positive chemotaxis in *O. marina* (Table 1b). Even after washing and transfer of *O. marina* back into $NH_4^+$- free seawater, measureable motile chemosensory responses towards a greater range of arginine test solutions (10-1000µM) were only recovered in cells that had not been incubated in $NH_4Cl$ solutions greater than 5µM (Table 1b). It appears that brief (i.e.

3 hour) exposure to low (<5µM) concentrations of $NH_4^+$ has reversible effects on the motile chemosensory responses of *O. marina*. Conversely, incubation in higher (>5µM) concentrations and/or chronic exposure to $NH_4^+$ (e.g. regenerated extracellular $NH_4^+$ in batch-cultures) can result in irreversible impairment of chemotaxis in *O. marina*.

The sensory apparatus (chemoreceptors, signal transduction pathways etc) that regulates chemotaxis in *O. marina* still requires fuller investigation. However, a consideration of the mechanisms that control chemotaxis in the much-studied freshwater ciliate *Paramecium tetraurelia* provides some potential insights into results from experimental work with *O. marina*. In *Paramecium*, motile chemosensory responses are mediated by membrane electrical changes; chemoattractants elicit membrane hyperpolarisation and positive chemotaxis while repellents cause depolarisation and negative chemotaxis (30). Some chemoattractants (e.g. glutamate) elicit chemotaxis after binding with specific cell-surface chemoreceptors (31); other non-specific mechanisms of activation are under also being investigated. Pertinently, ammonium chloride ($NH_4Cl$) elicits positive chemotaxis in *Paramecium* although cell-surface receptors specific for $NH_4^+$ have not yet been elucidated (6). Instead it is suspected that alteration of intracellular pH is the likely mechanism by which $NH_4Cl$ induces chemotaxis in *Paramecium* (6). Briefly, in solution, $NH_4Cl$ is in equilibrium with the membrane permeant molecule ammonia ($NH_3$) which (on passing into the cell) triggers hyperpolarisation. It is entirely possible that the chemosensory responses elicited by $NH_4^+$ in *O. marina* (like those in *Paramecium*) are not receptor based. Indeed, diffusion of $NH_4^+$ across the membrane of *O. marina* might result in activation of any cellular apparatus implicated in the control of chemotaxis. Regardless of the mechanism through which $NH_4^+$ elicits chemotaxis, it is clear that its excitatory effect can render *O. marina* unable to detect alternative stimuli that may elicit their effects via similar (or more specific, receptor based) modes of action; results from the $NH_4Cl$ addition and removal experiment underscore this observation. Here, the stimulatory effect elicited by 'in situ' $NH_4^+$ concentrations of 5µM (or higher) completely masked responses to 100µM arginine (Table 1b).

That regenerated $NH_4^+$ appears to have such a marked effect on the strength and consistency of chemotaxis in *O. marina* raises some important considerations relating to the maintenance and use of batch-cultured cells for research. Firstly, because relatively low (e.g. 5µM) concentrations of $NH_4^+$ (note that concentrations of regenerated $NH_4^+$ in prey-deplete batch-cultures frequently exceed 100µM) affect cells, thorough investigations of chemotaxes and characterisation of any chemosensory apparatus that might exist in *O. marina* are likely to be very difficult to conduct using batch-cultured cells. In some batch-culture studies, there may be a 'window of chemotaxis' in which ambient culture conditions (low-prey, low-$NH_4^+$) are more conducive to quantifying and observing chemotaxis in *O. marina* (Fig. 1a). However, the temporal existence of this window will vary from culture to culture and will be dependent on i) prey abundance and type (photosynthetic versus non-photosynthetic prey), ii) predator abundance (rapid grazing versus slow grazing activity) and iii) prey quality (high C:N versus low C:N prey) all of which affect levels of $NH_4^+$-regeneration by *O. marina* (5). Moreover, while this study has focused on regenerated $NH_4^+$, it is important to note that other regenerated compounds (e.g. amino acids leaked from conspecifics) and/or biogenic exudates (including toxins) from prey species will also affect the physiology and chemotaxis of batch-cultured *O. marina*. Clearly, to minimise (or at least regulate) the physiological effects of extracellular chemical stressors, *O. marina* from chemostat cultures should be used if at all possible; standard recovery periods (following washing and transfer of *O. marina* between different culture environments) must also be employed.

It should be highlighted that the 'window of chemotaxis' is so named, primarily because it represents a window of opportunity in which an experimenter is most likely to be able to measure motile chemosensory responses in laboratory-cultured *O. marina*. However, the conditions necessary for this 'window' also occur in the natural marine environment. As depicted (Fig. 1a) the 'window of chemotaxis' corresponds to

prey-depleted, low-$NH_4^+$ conditions, where motile, vacuole-deplete (containing no or very few prey vacuoles) *O. marina* are present. These conditions would – for example - prevail i) at the tail-end of grazing activity on an algal bloom, and ii) when toxic, graze-resistant or non-palatable algal prey are present. In both instances, the presence of residual algal prey would result in the uptake (for photosynthesis) of regenerated $NH_4^+$ and thus low levels of extracellular $NH_4^+$. Pertinently, in such circumstances, *O. marina* would be vacuole-depleted, motile and perhaps more reliant on chemosensory mechanisms to help locate new (or more suitable) sources of prey. It is noteworthy that conditions outside of the 'window of chemotaxis' (Fig. 1b and 1c) might also occur naturally. For example, large, prey-saturated, *O. marina* containing multiple internal prey vacuoles (Fig. 1b) would be present during the early stages of grazing activity on a dense algal bloom. For prey-saturated *O. marina*, motile chemosensory responses (for the location of new prey) are perhaps unnecessary and (owing to decreased cell-motility) ineffective. Conversely, small, prey-deplete, highly motile *O.marina* (Fig. 1c) would predominate in prey-starved conditions (e.g. at the end of an algal bloom or in the oligotrophic open ocean). It is reasonable to suspect that localised high-$NH_4^+$, low-prey conditions (Fig. 1c.) might occur over short-term time frames in certain marine habitats (e.g. within rock pools or microscale aggregations of *O. marina*). However, in the oligotrophic open ocean, physical dispersion and biological (e.g. bacterial) $NH_4^+$-uptake processes are likely to dictate that the high concentrations of regenerated $NH_4^+$ seen in laboratory batch-cultures (Table 1a) would not persist in the longer-term.

Clearly, the results reported here underscore the need to treat any ecological assumptions based studies with batch-cultured *O. marina* with caution. As previously highlighted (19), the positive chemotaxis elicited by $NH_4^+$ in *O. marina* could facilitate population cohesion in prey depleted, low Reynolds number environments (e.g. the typical rock pool habitat of *O. marina* [7]) where $NH_4^+$ regenerated by conspecifics would accumulate in the absence of photosynthetic prey. It follows that in prey depleted environments *O. marina* populations would remain compacted as cells sense and respond to $NH_4^+$ and additional biogenic cues leaked from conspecifics. Conversely, under low - $NH_4^+$ conditions and/or when strong 'non-self' stimuli (e.g. exudates from a phytoplankton bloom) are encountered, *O. marina* populations may engage in more active foraging. Because cohesion maintained by regenerated compounds is likely to be strongest at the centre of a microbial population, the directional responses of cells at its periphery (those exposed to non-self signals) could guide others in a follow-the-leader manner towards new foraging areas. However, it should be noted that in the oligotrophic ocean, concentrations of $NH_4^+$ may not reach (or persist at) those observed in laboratory studies due to biological uptake processes and nutrient cycling within marine 'microbial loop' communities (3, 23).

Finally, a few points on the suitability of the microcapillary assay for studying motile chemosensory responses in *O. marina* should be noted. Indeed, the microcapillary assay can be useful for the investigation of motile chemosensory responses in general. For example, as predatory cells approach prey items, they pass through gradients of excreted metabolites (a zone termed the phycosphere – [25]). The microcapillary technique simulates 'phycosphere' conditions to some degree; however it cannot be used to recreate more discrete chemical gradients or near-field biochemical changes (e.g. chemical pulses) that are suspected to be associated with the presence of prey items (20, 32). Importantly, because (in this and other studies [19, 27]) the microcapillary assay is used to quantify chemoresponses to chemical gradients after relatively long (e.g. 2 minute) integration periods, it is not possible to infer the effects of test stimuli on chemosensory behaviour over more immediate (micro/millisecond) timescales. On this point, it is perhaps noteworthy that in the $NH_4Cl$ addition and removal study reported here (Table 1b), no *O. marina* were observed (at $t_2$min) inside control tubes (containing $NH_4^+$-free seawater) when 'in situ' concentrations of $NH_4^+$ exceeded 5µM. Conversely, when ambient $NH_4^+$ was non-detectable (typically following washing and transfer of *O. marina* to $NH_4^+$-free

seawater), the number of *O. marina* observed inside control tubes at $t_2$min increased to between 8 and 15. This observation suggests that *O. marina* does indeed respond to discrete chemical gradients, and that (once adjusted to ambient conditions) will orientate towards similar (or away from dissimilar) conditions - perhaps to help maintain intracellular homeostasis. Given these considerations, use of the microcapillary assay to investigate stimuli (e.g. algal toxins) that might be expected to elicit negative chemotaxis in the natural marine environment is fundamentally flawed. The failure of *O. marina* to enter microcapillary tubes may not be indicative of an avoidance response or true negative chemotaxis but rather the affinity of cells for dissolved stimuli (e.g. regenerated $NH_4^+$) present in the extracellular batch-culture environment. Novel methodologies, particularly those designed for the study of cellular responses to near-field chemical signals, are now required.

## ACKNOWLEDGEMENTS

The author wishes to thank Professor Kevin J Flynn (Swansea University, UK) and the Natural Environmental Research Council (NERC, UK).

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